

Skeletal muscle specification by myogenin and Mef2D via the SWI/SNF ATPase Brg1

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Myogenin is required not for the initiation of myogenesis but instead for skeletal muscle formation through poorly understood mechanisms. We demonstrate in cultured cells and, for the first time, in embryonic tissue, that myogenic late genes that specify the skeletal muscle phenotype are bound by MyoD prior to the initiation of gene expression. At the onset of muscle specification, a transition from MyoD to myogenin occurred at late gene loci, concomitant with loss of HDAC2, the appearance of both the Mef2D regulator and the Brg1 chromatin-remodeling enzyme, and the opening of chromatin structure. We further demonstrated that ectopic expression of myogenin and Mef2D, in the absence of MyoD, was sufficient to induce muscle differentiation in a manner entirely dependent on Brg1. These results indicate that myogenin specifies the muscle phenotype by cooperating with Mef2D to recruit an ATP-dependent chromatin-remodeling enzyme that alters chromatin structure at regulatory sequences to promote terminal differentiation.

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Introduction

Extensive analyses of skeletal muscle differentiation have shown that myogenic regulatory factors (MRFs) of the MyoD family are involved in skeletal muscle development. One of the first clues to understanding the control of muscle development at the molecular level came from the discovery of MyoD, a DNA-binding transcriptional activator capable of reprogramming cultured fibroblasts such that they resembled skeletal muscle cells molecularly and morphologically (Davis *et al*, 1987). Two other MRF family members, Myf5 and Mrf4, also were shown to induce skeletal muscle differentiation upon ectopic expression in fibroblast cells (Edmondson and Olson, 1989; Miner and Wold, 1990; Yutzey *et al*, 1990). Interestingly, mice deficient for MyoD, Myf5, or Mrf4

revealed no significant defect in skeletal myogenesis (Braun *et al*, 1992; Rudnicki *et al*, 1992; Braun and Arnold, 1995; Patapoutian *et al*, 1995; Zhang *et al*, 1995); however, analyses of double mutants indicated that these factors showed functional redundancy (Arnold and Winter, 1998; Kassam-Duchossoy *et al*, 2004).

In vivo experiments suggest a different functional role for myogenin, a fourth member of the MRF family. Myogenin-deficient mice showed normal myoblast formation during embryogenesis, but were not viable because of a deficiency during the later stages of skeletal muscle development (Hasty *et al*, 1993; Nabeshima *et al*, 1993; Rawls *et al*, 1995; Venuti *et al*, 1995). These studies indicated that myogenin is critical in the late phase of skeletal muscle differentiation. However, unlike the other MRFs, myogenin itself inefficiently induces skeletal muscle differentiation in culture (Gerber *et al*, 1997; Bergstrom and Tapscott, 2001; Roy *et al*, 2002). The molecular basis controlling the contribution of myogenin to specification of the skeletal muscle phenotype remains unclear.

The Mef2 family of transcriptional regulators also has a critical role during myogenesis (Molkentin *et al*, 1995). However, Mef2 family members are expressed in most tissues. Mef2D has a skeletal muscle-specific isoform that arises due to alternative splicing (Martin *et al*, 1994), and Mef2D and MyoD can synergistically accelerate skeletal muscle differentiation in culture (Penn *et al*, 2004). Mef2A and Mef2C have also been shown to contribute to the induction of skeletal muscle differentiation in culture (Black *et al*, 1995; Molkentin *et al*, 1995; Naidu *et al*, 1995). However, Mef2 proteins alone are not capable of directing skeletal muscle differentiation in the absence of MRFs (Molkentin *et al*, 1995).

A key advance in understanding skeletal muscle development was recognition of the interplay between myogenic regulatory proteins that initiate the differentiation-specific gene expression program and chromatin-modifying enzymes that alter chromatin structure at the loci to be expressed. Acetyltransferases interact with MyoD and acetylate promoter histones, as well as MyoD itself (reviewed in Berkes and Tapscott, 2005). Mef2 interacts with histone deacetylase enzymes to repress differentiation in immortalized myoblasts (Lu *et al*, 2000). The ATP-dependent chromatin-remodeling enzyme SWI/SNF interacts with both MyoD and Mef2 proteins and is required for muscle differentiation via its ability to alter chromatin structure at endogenous, muscle-specific loci (de la Serna *et al*, 2001a, 2005; Simone *et al*, 2004).

To understand the relationship between muscle-specific transcription factors and chromatin-remodeling enzymes, we analyzed the activation of late myogenic genes that specify skeletal muscle structure and function in developing embryos as well as in defined cell culture models. Expression of myogenic late genes signifies commitment to the development of muscle tissue, but the precise mechanisms that specify myogenic commitment are not well defined. Here, we definitively demonstrate that myogenin and Mef2D

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cooperate to determine the skeletal muscle phenotype through recruitment of SWI/SNF chromatin-remodeling enzymes. Furthermore, the data suggest that the temporal regulation of early and late myogenic gene expression is regulated by recruitment of SWI/SNF enzymes by different myogenic regulatory proteins at different stages of skeletal muscle differentiation.

Results

Chromatin remodeling by SWI/SNF complex mediates the late stages of myogenesis

The MRF and MEF2 families of proteins have been reported to play a critical role in myogenesis (Davis *et al*, 1987; Rudnicki *et al*, 1993; Molkenkin *et al*, 1995). These families of proteins are detectable in the myotome and are present throughout the development of the skeletal muscle lineage during embryogenesis (Sassoon *et al*, 1989; Bober *et al*, 1991; Hinterberger *et al*, 1991; Ott *et al*, 1991; Edmondson *et al*, 1994). However, it is not clear how expression of muscle-regulatory proteins is

related to chromatin-remodeling and transcriptional activation of the genes that specify the muscle phenotype during myogenesis in the embryo. To address this question, we first confirmed that, in the developing embryo, late myogenic marker genes such as muscle creatine kinase (MCK) and desmin could be detected from 12.5 d.p.c., when the primary muscle fibers begin to form, and that expression was dramatically increased at 14.5 d.p.c., at which point some myotube formation has already occurred (Figure 1A; Furst *et al*, 1989; Venuti *et al*, 1995). A quantitative restriction enzyme accessibility assay performed using embryonic tissue revealed that the increased chromatin accessibilities of both the MCK and desmin promoters were coincident with the induction of mRNA accumulation (Figure 1B). Therefore, we evaluated whether the change in chromatin structure was related to an increase in the expression of the *MRF* or *Mef2* genes or to changes in levels of subunits of the SWI/SNF chromatin-remodeling complex, which mediates chromatin remodeling of myogenic genes in cell culture (de la Serna *et al*, 2001a). MyoD and Myf5 have been shown to play critical but

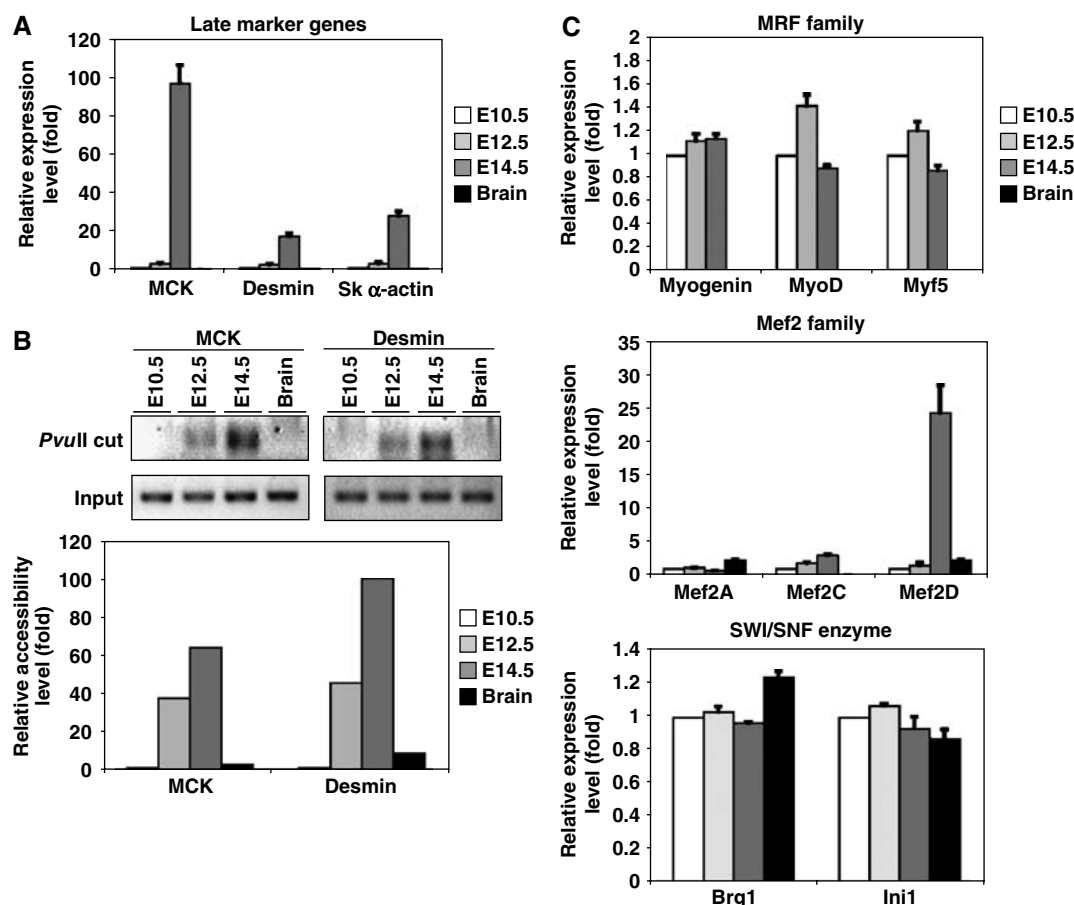


Figure 1 The expression of myogenic late marker genes correlates with chromatin remodeling on the promoters of myogenic late marker genes in developing embryos. (A) Time course of late myogenic marker gene expression during embryogenesis. mRNA levels of each gene were examined at the indicated stage by quantitative RT-PCR. Late marker genes: MCK, desmin, and α -skeletal actin. Fold induction was standardized to the E10.5 stage point of myogenic tissue, which was defined as 1. (B) Chromatin accessibility at the endogenous late marker genes correlates with the onset of gene expression. Nuclei were isolated from each indicated embryonic tissue. A modified LM-PCR protocol (de la Serna *et al*, 2005) was utilized to visualize digested genomic DNA. The PCR product is shown as an inverse image (top panel). Relative accessibility was standardized to accessibility of the E10.5 sample, which was defined as 1. Quantification represents the average of two independent experiments. Variation between experiments did not exceed 20% for any sample. (C) Time course of regulatory protein gene expression during embryogenesis. mRNA levels of each gene were examined at the indicated stage by quantitative RT-PCR and quantified as described above. MRF family: MyoD, Myogenin, and Myf5. Mef2 family: Mef2A, Mef2C, and Mef2D. SWI/SNF enzyme subunits: Brg1 and Ini1.

redundant roles in early myogenesis (Rudnicki *et al*, 1993), whereas myogenin has been linked to the regulation of the later phases of myogenesis (Hasty *et al*, 1993; Nabeshima *et al*, 1993). A quantitative reverse transcriptase (RT)-PCR assay showed that MyoD, Myf5, and myogenin mRNAs were expressed at 10.5 d.p.c. and that expression did not change significantly through E14.5 (Figure 1C). The transcript levels of the SWI/SNF components Brg1 and Inr1 also did not change during this time span. In contrast, Mef2C and Mef2D mRNAs were upregulated at 12.5 d.p.c., when late marker gene expression initiated, and Mef2D was dramatically upregulated by 14.5 d.p.c. (Figure 1C).

To determine which factors are involved in chromatin-remodeling and gene activation during late myogenesis, we used embryonic tissue to perform chromatin immunoprecipitation (ChIP) assays on MCK and desmin regulatory sequences (Figure 2B). Both the proximal promoter region and enhancer sequences for each gene were examined in these and subsequent ChIP experiments; no difference in factor binding to promoters versus enhancers was observed. MyoD was present on late gene promoters and enhancers at E10.5 even though the genes were transcriptionally inactive and had not yet undergone changes in promoter accessibility. Class I HDACs are known to associate with MyoD to prevent transcriptional activation (Mal *et al*, 2001; Puri *et al*, 2001; Mal and Harter, 2003); further analysis demonstrated that HDAC2, but not HDAC1, was also present at the late gene loci (Figure 2B and data not shown). At E12.5, when late gene expression and chromatin remodeling begins, a decrease in MyoD and HDAC2 binding was observed at the MCK and desmin loci, concomitant with an increase in myogenin, Mef2, and Brg1 binding. At E14.5, when late gene expression is dramatically induced, myogenin, Mef2, and Brg1 remained associated with the MCK and desmin regulatory sequences. As the antibody against Mef2 recognizes the A, C, and D

isoforms, we cannot identify the specific isoform present at these sequences. However, the expression data (Figure 1C) strongly suggest that the isoform present is most likely Mef2D.

These data indicate that, in developing embryonic muscle tissue, inactive late gene loci are first bound by MyoD and HDAC2 prior to chromatin-remodeling and transcriptional activation. Chromatin-remodeling and transcription initiation are correlated with the disappearance of MyoD and HDAC2 from the late gene regulatory sequences and the coincident appearance of the myogenin and Mef2D regulatory proteins, as well as the Brg1 chromatin-remodeling enzyme. This temporal pattern of factor interactions strongly suggests that myogenic late gene expression during embryogenesis is specifically mediated not by MyoD, but by myogenin and Mef2D, in a manner that involves chromatin remodeling by Brg1-based SWI/SNF enzymes.

Myogenic late gene expression is delayed until myogenin and Mef2 can target Brg1-based chromatin-remodeling activity to late gene promoters

To directly evaluate the contributions of MRFs and SWI/SNF chromatin-remodeling enzymes in the activation of late myogenic marker genes, we examined MyoD-directed differentiation of B22 cells, which are NIH3T3 fibroblasts that contain a tetracycline suppressible dominant-negative BRG1 (de La Serna *et al*, 2000). Temporal analysis of myogenic gene expression in these cells revealed that induction of the early myogenin gene at 4 h post-differentiation preceded activation of the late MCK and desmin genes at 8 h post-differentiation (Figure 3A). As was observed in embryonic skeletal muscle tissue, induction of Mef2D correlated with induction of the late gene markers. Each of these gene induction events was inhibited by the expression of an ATPase-deficient, dominant-negative BRG1 protein (minus tet samples; Figure 3A),

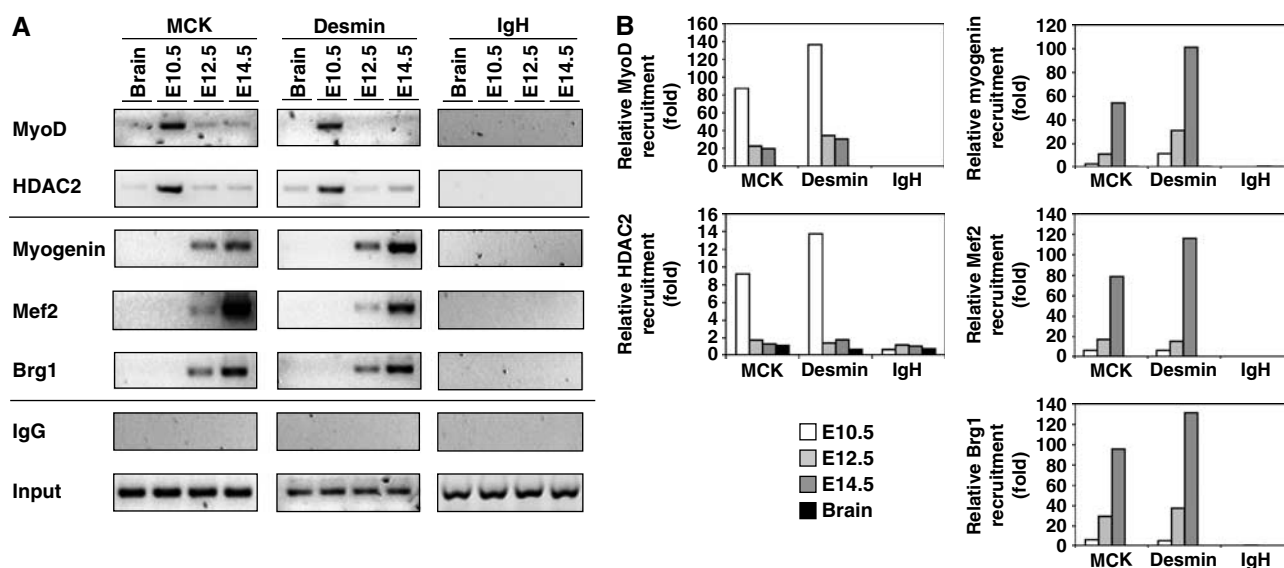


Figure 2 Time course of factor interactions with myogenic promoters (as measured by ChIP) indicates that myogenin, Mef2, and Brg1 are recruited onto late muscle marker regulatory sequences at the time of gene expression. (A) ChIP assays for MyoD, HDAC2, Myogenin, Mef2, and Brg1 were performed at the indicated stages of embryonic development. (B) Quantified ChIP data. Relative recruitment was defined as the ratio of amplification of the PCR product relative to 1% of input genomic DNA. Values obtained from brain tissue were defined as 1. Quantification represents the average of two independent experiments. Variation between experiments did not exceed 20% for any sample. The IgH enhancer region, which contains an E box that does not bind MyoD, was amplified as control for ChIP specificity.

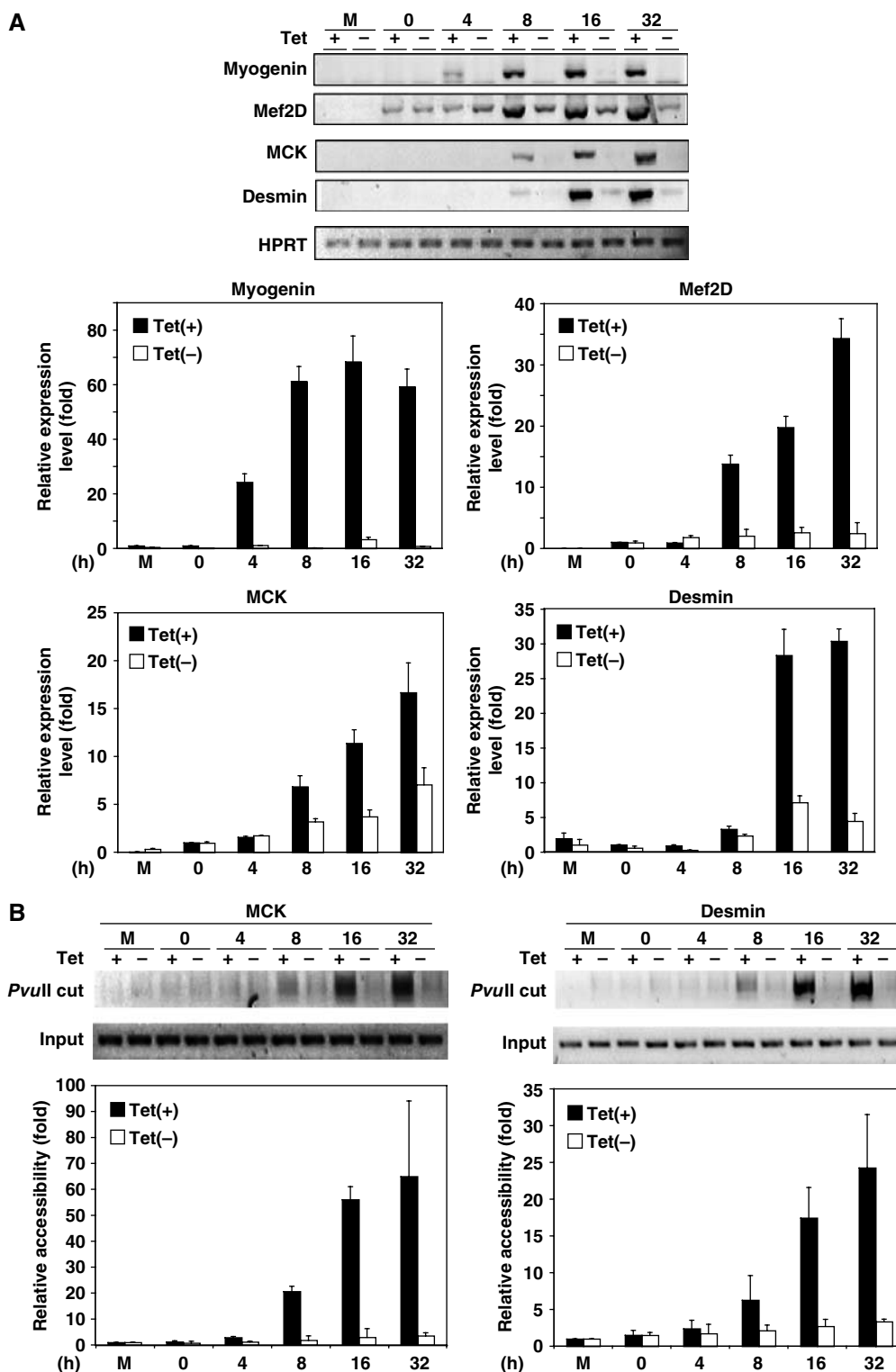


Figure 3 (A) Time course of myogenin, Mef2D, and late marker gene expression during MyoD-induced differentiation of B22 cells. Cells grown in the absence of tetracycline (tet), which express dominant-negative BRG1, or in the presence of tet, which do not express dominant-negative BRG1, were infected with retrovirus containing MyoD. After 30 h, differentiation was initiated by replacement of the media with a low serum differentiation media (time 0). mRNA levels of each gene were determined at the indicated time points by RT-PCR. Fold induction was standardized to the 0-h time point, which was set at 1. (B) Restriction enzyme accessibility increases at endogenous late marker loci at the time of gene expression and requires functional Brg1. Relative accessibility was defined as the ratio of the level of cleaved genomic DNA in a given sample relative to the input in the same sample and standardized to the mock, which was set at 1. Cleaved genomic DNA was visualized as described in Figure 1.

confirming the necessity of chromatin-remodeling enzymes for late gene expression (de la Serna *et al*, 2001a, b). *In vivo* restriction enzyme accessibility experiments showed an

increase in digestion of sites on both the MCK and desmin promoters starting 8 h after the onset of differentiation, and a further increase in accessibility was observed 16 h after

differentiation (Figure 3B). Thus, changes in late gene promoter accessibility directly correlated with the onset of late gene expression, again reflecting events occurring in embryonic skeletal muscle. These changes in late gene promoter accessibility occurred later than the changes in chromatin remodeling on the myogenin promoter in the same culture system (de la Serna *et al*, 2005), and all changes in chromatin accessibility were completely dependent on functional Brg1.

We next performed ChIPs to detect the recruitment of factors onto the promoters and enhancers of late myogenic markers (Figure 4). We observed that the temporal order of factor interactions in differentiating cells reflected that in developing embryos. MyoD and HDAC2 were bound to the MCK and desmin regulatory sequences at the onset of differentiation in a manner independent of functional Brg1-based SWI/SNF enzymes, however, levels of these proteins were declining by 8 h post-differentiation, when late gene expression initiated (Figure 3A), and were present at background or at very low levels by 16 h post-differentiation. The release of MyoD and HDAC2 correlated with the recruitment of the myogenin and Mef2 proteins at 8 h post-differentiation. Brg1 was recruited with the same kinetics as myogenin and Mef2, providing a likely explanation for why the early binding of MyoD did not require Brg1. Interestingly, the ability of the dominant-negative BRG1 protein to interact with the late gene promoters was compromised, suggesting that the presence of MyoD was not sufficient to target Brg1. Instead, induction of myogenin and Mef2D by MyoD and the subse-

quent binding of myogenin and/or Mef2 may be critical for targeting Brg1 and SWI/SNF enzyme to the late gene promoters. These results contrast with previous characterization of factor binding at the myogenin promoter, which indicated that the dominant-negative BRG1 could be targeted to the promoter by the MyoD regulator (de la Serna *et al*, 2005).

To further investigate the relationship between Brg1, Mef2 and myogenin, we performed re-ChIP assays (Figure 5A). Immunoprecipitation of Brg1 confirmed its association with late gene regulatory sequences starting at 8 h post-differentiation. Subsequent re-precipitation experiments revealed the simultaneous presence of myogenin and Mef2 on Brg1-bound sequences. As observed in Figure 4, binding by each factor required functional Brg1, as functional Brg1 was required for the induction of both myogenin and Mef2.

Finally, we utilized coimmunoprecipitation techniques to determine whether Brg1 was associated with Mef2 and/or myogenin during myogenesis. In differentiated cells, we observed coimmunoprecipitation between endogenous Brg1 and endogenous Mef2 and endogenous myogenin. No interactions were observed in mock-differentiated cells that were not expressing MyoD, or in MyoD-expressing cells that were harvested before the induction of differentiation ($t=0$ samples). The lack of significant coprecipitation between Mef2, myogenin, and mutant Brg1 in the minus tetracycline samples likely reflects the minimal induction of Mef2 and myogenin in the absence of functional Brg1 (Figure 3A; de la Serna *et al*, 2001a,b) as opposed to an inability of these

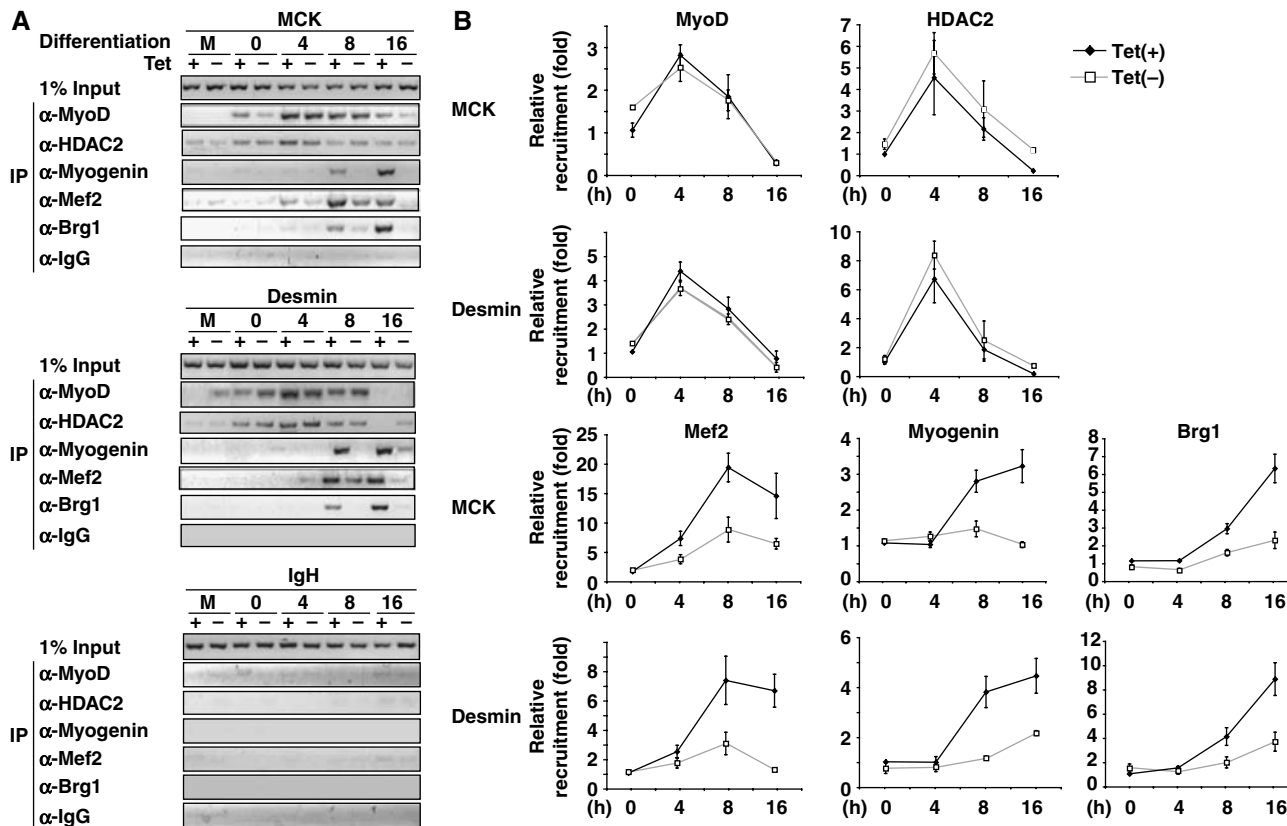


Figure 4 MyoD binds to late gene regulatory sequences, but myogenin, Mef2, and Brg1 are recruited at the time of late gene expression. (A) ChIPs were performed with antibodies against the indicated proteins or with nonspecific IgG on mock or MyoD-differentiated samples as indicated. (B) Quantification of the levels of MyoD, HDAC2, myogenin, Mef2, and Brg1 present at the MCK and desmin loci by ChIP analysis. Band intensities in each lane were normalized to input. The data reflect the mean \pm standard deviation from three independent experiments.

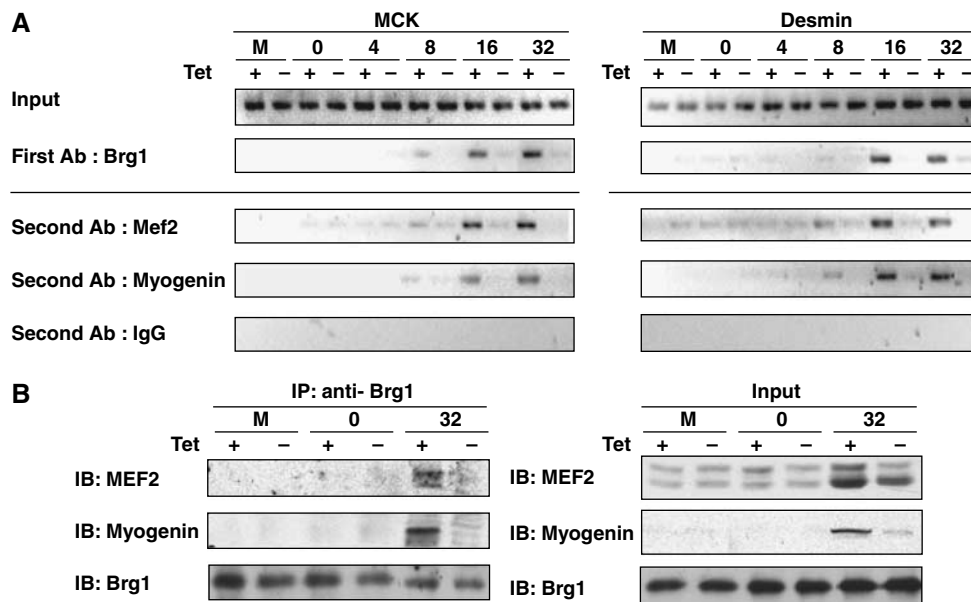


Figure 5 Brg1 associates with myogenin and Mef2. **(A)** Brg1 corecruitment with myogenin and Mef2 at the MCK and desmin promoters by Re-ChIP during a time course of MyoD-induced differentiation. Re-ChIP experiments first used an antibody against Brg1, then a second antibody against myogenin, Mef2, or nonspecific IgG. 1% of input is shown. **(B)** Endogenous Brg1 physically interacts with endogenous Mef2 and myogenin during myogenesis. As an input control, 10% of cell lysate utilized was immunoblotted with the same antibodies.

proteins to associate with ATPase-deficient Brg1. These data suggest that Brg1 is targeted to late genes via the Mef2 and myogenin proteins.

Collectively, the data indicate that the pattern of factor recruitment on late gene regulatory sequences in MyoD-differentiated cells mimics that in embryonic skeletal muscle tissue. In both systems, myogenic late gene expression is delayed until myogenin is expressed and Mef2 is induced, and the two proteins can be recruited coordinately to late gene loci with Brg1 to promote chromatin-remodeling and transcription initiation. This order of events is in contrast to regulation of the myogenin gene, where MyoD recruits Brg1 onto the myogenin promoter (Simone *et al*, 2004; de la Serna *et al*, 2005). These data suggest that part of the distinction between early and late myogenic gene expression is due to the sequential recruitment of Brg1-based SWI/SNF chromatin-remodeling enzymes to different genes by different MRFs.

Mef2D and myogenin synergistically induce myogenesis in a Brg1-dependent manner

Myogenin and Mef2D levels are dramatically induced during myogenesis in the embryo and in cell culture models of myogenic differentiation (Figures 2A and 3A), and the data presented above indicate that both are critical for the activation of myogenic late gene expression. These data raise the hypothesis that myogenin and Mef2D may be able to efficiently induce myogenic differentiation in the absence of MyoD. To probe mechanisms of cooperation between Mef2D and myogenin and to investigate the ability of Mef2D and myogenin to potentiate myogenesis, we coexpressed myogenin and the muscle-specific isoform of Mef2D in the B22 cell line in the presence or absence of dominant-negative BRG1. Introduction of MyoD has long been recognized to drive the myogenic differentiation programme in nonmuscle cells, but NIH3T3-derived cells do not complete

the programme by fusing to form myotube-like fibers. Coexpression of MyoD and Mef2D accelerates myogenic differentiation (Penn *et al*, 2004); however, expression of myogenin alone is insufficient to efficiently promote myogenesis (Gerber *et al*, 1997; Bergstrom and Tapscott, 2001; Roy *et al*, 2002).

As expected, introduction of MyoD induced the expression of myosin heavy chain (MyHC), another late gene, in a Brg1-dependent manner, but did not change cellular morphology (Figure 6A and Supplementary Figure 1). Surprisingly, cointroduction of myogenin and Mef2D in the absence of introduced MyoD not only induced MyHC expression in a Brg1-dependent manner but also induced myotube formation (Figure 6A and Supplementary Figure 1). Myotube formation was not a rare event; 90% of nuclei were present in multinucleated cells (data not shown). Myogenin or Mef2D alone did not induce MyHC expression or morphology changes, and, as expected, introduction of MyoD plus Mef2D activated MyHC and promoted myotube formation (Figure 6A) because MyoD activates endogenous myogenin expression. Introduction of all three muscle regulatory factors, MyoD, myogenin, and Mef2D, generated similar results (Figure 6A and Supplementary Figure 1). The generality of these results was confirmed by demonstrating that myogenin and Mef2D could induce myogenic late gene expression from endogenous loci when introduced together, but not separately, into 3T3-L1 preadipocyte cells (Supplementary Figure 2). These data conclusively demonstrate that myogenin and Mef2D have the potential to induce myogenic differentiation without MyoD and have the additional ability to induce morphological changes in 3T3 cell structure that are associated with myogenesis, a property not shared by MyoD.

In these experiments, the known inability of MyoD to promote morphological changes in differentiated, NIH3T3-based cells allowed us to demonstrate that myogenin and

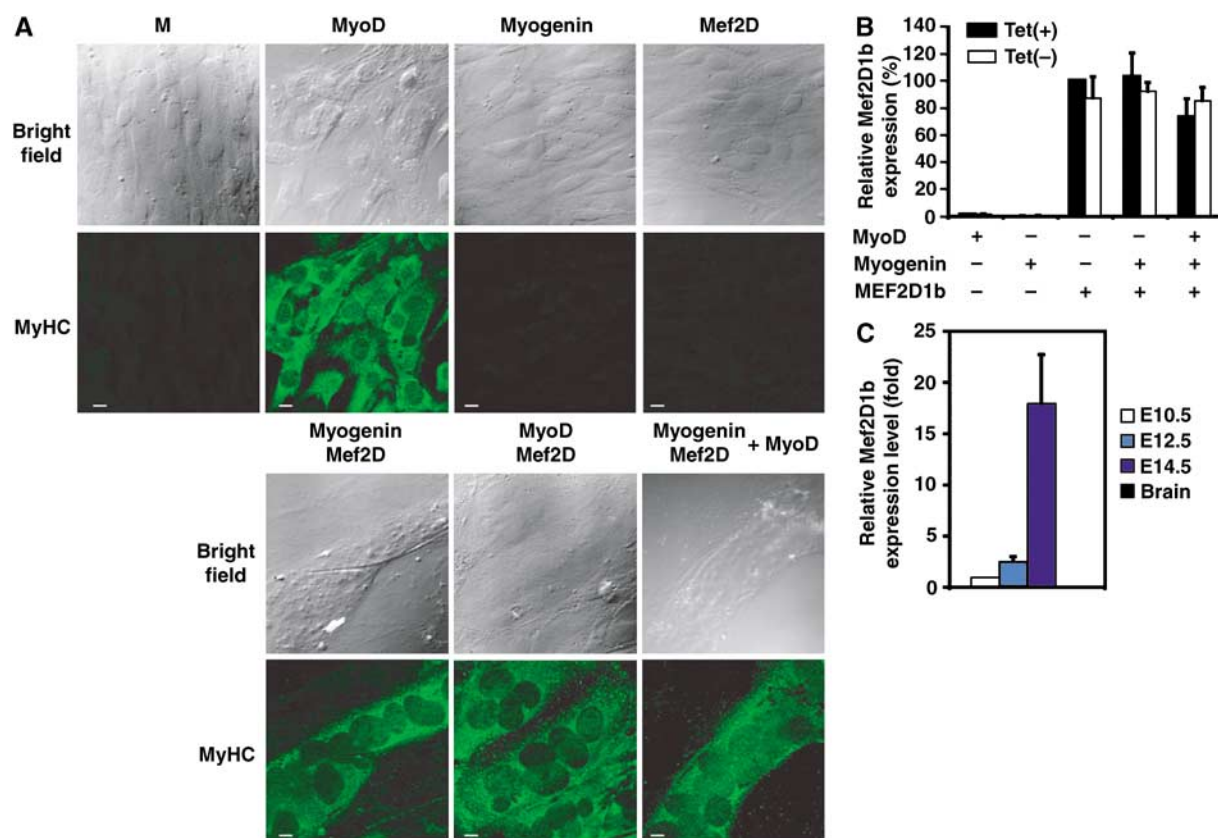


Figure 6 Myogenin and Mef2D1b are sufficient to induce skeletal muscle differentiation and myotube formation. (A) Immunostaining for MyHC in B22 cells that had been cultured in the presence of tetracycline and either mock (M) infected or infected with retroviruses encoding MyoD, myogenin, and/or Mef2D1b, as indicated. Following infection, cells were differentiated for 36 h and then stained with anti-MyHC antibody. Bright field images are also shown. White scale bar—10 μ m. (B) *Mef2D1b* gene induction after infection of B22 cells grown in the presence or absence of tet with retroviruses expressing the indicated myogenic factor(s). The expression of Mef2D1b in Mef2D1b-infected cells plus tet was defined as 100%. The data are the mean plus/minus standard deviation from three independent experiments. (C) Time course of Mef2D1b expression during embryogenesis. Mef2D1b mRNA levels were examined at the indicated stage by quantitative real-time PCR. Fold induction was standardized to the E10.5 stage.

Mef2D promote the complete pattern of late gene expression, including the events that lead to myotube formation. However, MyoD induces the expression of both myogenin and Mef2D, which raises the question of why NIH3T3 cells are blocked for myotube formation at the end of myogenesis. The answer lies in the ability of both cultured cells and developing embryonic muscle cells to express Mef2D1b, the muscle-specific isoform of Mef2D (Martin *et al*, 1994). MyoD differentiation of NIH3T3-based cells showed no induction of the muscle-specific Mef2D1b isoform (Russo *et al*, 1998; Figure 6B). In contrast, RT-PCR analysis of RNA from embryonic tissue using Mef2D1b-specific primers showed that the Mef2D induced beginning at E12.5 and highly induced by E14.5 is the Mef2D1b isoform (Figure 6C). Myotube formation in the presence of myogenin and Mef2D (Figure 6A) was achieved because we were introducing the Mef2D1b isoform. The basis by which the structural differences between Mef2D isoforms relate to function has not been further examined.

Myogenin/Mef2D induce skeletal muscle differentiation by promoting Brg1-mediated chromatin remodeling

To analyze the mechanisms responsible for the synergistic activity of myogenin/Mef2D in promoting myogenesis, we evaluated the molecular consequences of coexpression of

these regulatory proteins. Expression of each of the exogenously introduced regulatory factors was confirmed by Western blot (data not shown). Coexpression of myogenin and Mef2D1b did not activate the endogenous *MyoD* gene, as measured by quantitative real-time PCR, but did induce expression from the endogenous MCK and desmin loci (Figure 7A). Further analyses indicated that the combination of myogenin and Mef2D could promote targeting of Brg1 to both late gene regulatory sequences (Figure 7B) and facilitate changes in restriction enzyme accessibility at late gene regulatory sequences (Figure 7C). These observations explain how ectopic expression of myogenin and Mef2D induces skeletal muscle-specific gene expression and differentiation.

We also note that MyoD could recruit dominant-negative Brg1 to the myogenin promoter, but not to late gene regulatory sequences (Figures 4 and 5; de la Serna *et al*, 2005). In contrast, myogenin and Mef2D were capable of recruiting dominant-negative Brg1 to late gene sequences (Figure 7B). These data further reinforce the idea that Mef2D and myogenin promote the association of Brg1 with late gene loci. Finally, the data indicate that late gene expression induced by ectopic expression of myogenin and Mef2D required functional Brg1 (Figures 6 and 7), definitively demonstrating that Brg1 was not solely required for the induction of myogenin and Mef2D, but instead was also required for changes in

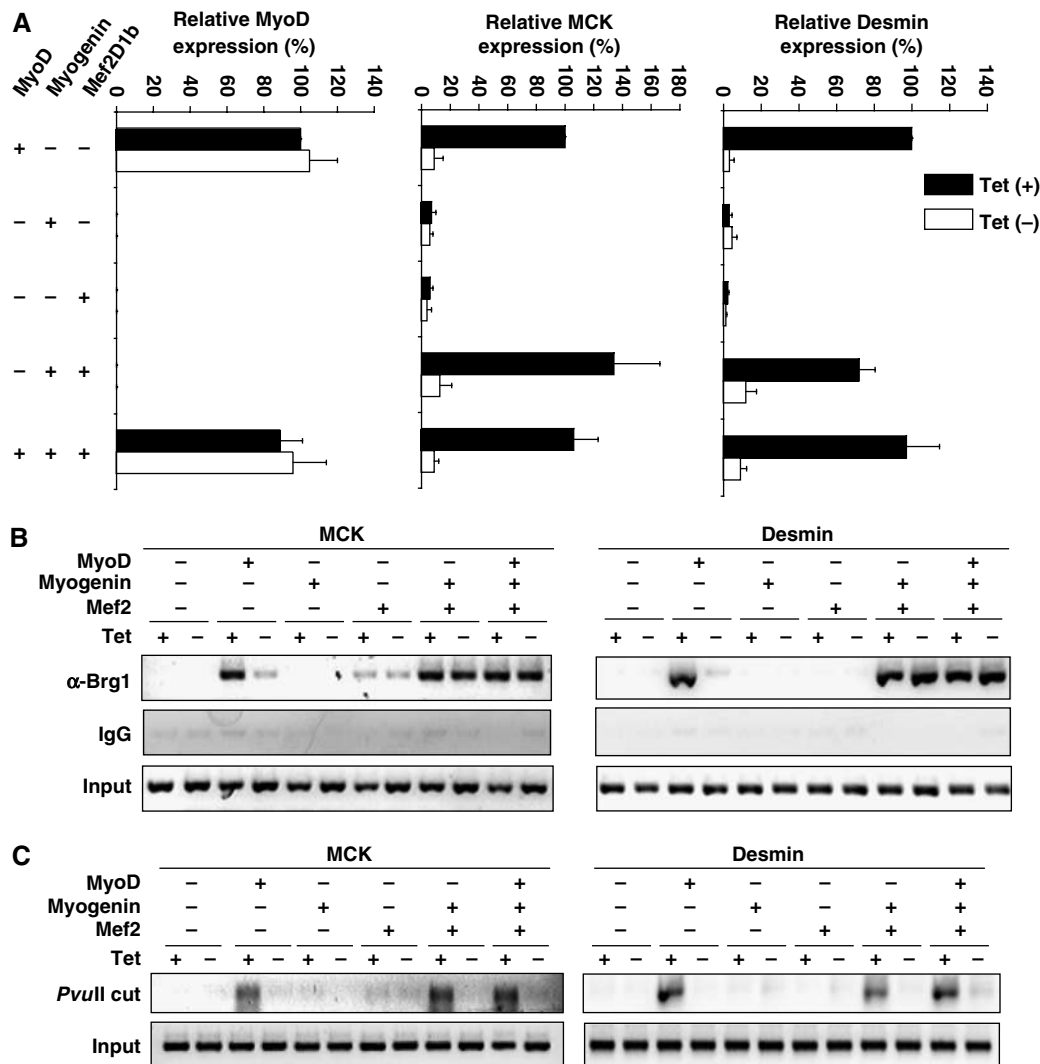


Figure 7 Myogenin and Mef2D1b induce skeletal muscle differentiation and myotube formation via recruitment of Brg1 and chromatin-remodeling activity. (A) Coexpression of myogenin and Mef2D induces endogenous late gene expression, but does not induce MyoD expression. B22 cells expressing or not expressing dominant-negative BRG1 were infected with the indicated retroviruses and allowed to differentiate for 30 h. mRNA levels of each gene were examined by quantitative real-time PCR. Fold induction was presented relative to the levels present in the MyoD-infected, plus tet sample. (B) Coexpression of myogenin and Mef2D1b recruits Brg1 to late gene loci. ChIPs were performed as described in Figure 3 on cells that were infected with retroviruses expressing the indicated proteins and that were differentiated for 36 h. (C) Coexpression of myogenin and Mef2D1b increases chromatin accessibility at late gene regulatory sequences in a Brg1-dependent manner. Cleaved genomic DNA was detected by modified LM-PCR as described in Figures 1 and 2.

chromatin structure at late gene regulatory sequences. We conclude that the loss of late marker gene expression in the absence of functional Brg1 is caused both by a lack of induction of myogenin and Mef2D, as well as by a loss of recruitment of SWI/SNF enzyme to late genes via myogenin/Mef2.

Discussion

Analyses of MRF function by mouse genetic studies have revealed that myogenin has a potentially unique role during skeletal muscle differentiation that is not shared by MyoD or the other family members (Rawls *et al*, 1995). These data indicate that myogenin is involved in the later steps of skeletal muscle differentiation, but the molecular basis for this ability has not been determined previously. Here we present evidence demonstrating how myogenin specifies the

skeletal muscle phenotype in both developing embryos as well as in a tissue culture model for differentiation. Myogenin cooperates with the Mef2D muscle-specific isoform and Brg1-based SWI/SNF chromatin-remodeling enzymes to alter chromatin structure at myogenic late gene promoters. This facilitates expression of the genes encoding the structural proteins and enzymes needed to specify skeletal muscle and the subsequent morphological changes that promote myotube formation.

Demonstration that myogenin and Mef2D specify the skeletal muscle phenotype

Our analysis of factor binding to myogenic late gene promoters in developing embryonic skeletal muscle indicates that, though MyoD binds to these promoters, late gene expression correlates with the replacement of MyoD by myogenin and the appearance of Mef2D, the SWI/SNF ATPase, Brg1, and

changes in nuclease accessibility at late gene promoters that strongly suggest an opening of promoter chromatin structure. To our knowledge, these are the first quantitative assessments of gene expression, factor binding, and chromatin accessibility in embryonic skeletal muscle tissue, and thus we have identified the relevant regulators and defined the temporal order of events that facilitate myogenic late gene expression and differentiation *in vivo* in the developing embryo.

The presence of MyoD at late gene loci prior to gene activation correlates with the simultaneous presence of HDAC2, a class I histone deacetylase. Though there are no reports of HDAC interaction with myogenic late gene loci, previous work has indicated that MyoD can bind to HDAC1 in C2C12 myoblasts (Mal *et al*, 2001; Puri *et al*, 2001), and one report demonstrated by ChIP that MyoD and HDAC1 were present at the myogenin promoter in C2C12 myoblasts and that HDAC1 binding was lost upon differentiation (Mal and Harter, 2003). Furthermore, treatment of C2C12 myoblasts with HDAC inhibitors activated the expression of MyHC and other late genes and promoted myoblast fusion (Iezzi *et al*, 2004), demonstrating the functional relevance of HDAC in suppressing late gene expression. Our data are consistent with these previous reports showing that there are HDAC-mediated mechanisms through which the activity of MyoD can be suppressed.

Although it has long been recognized that Mef2 family members and MyoD family members can cooperatively activate myogenic reporter genes as well as endogenous myogenic gene expression (Molkentin *et al*, 1995), there has been little understanding of whether and how specific family members of each family control the temporal expression of particular myogenic genes. Having identified myogenin and Mef2D as the relevant molecules present at the time of myogenic late gene activation in developing embryos, we turned to a cell culture model for skeletal myogenesis to examine the sufficiency of myogenin and Mef2D in promoting skeletal muscle formation. Each of the observations made in developing embryonic tissue was recapitulated in a tissue culture model in which ectopic expression of myogenic regulatory proteins drives the trans-differentiation of fibroblast cells. The observation that ectopic expression of myogenin and Mef2D, in the absence of MyoD, initiates myogenic differentiation and promotes the same temporal order of regulatory factor interactions and functions at late gene promoters that was seen in the developing embryo indicates that myogenin and Mef2D are sufficient among the muscle regulatory factors to specify skeletal muscle differentiation.

Ironically, it was an inherent limitation of the particular fibroblast line being used in our experiments that helped identify the contribution of the Mef2D1b protein, the muscle-specific isoform of Mef2D. Trans-differentiation of NIH3T3-derived cells by MyoD induced endogenous myogenin and Mef2D proteins, which, along with the Brg1 ATPase, localized to the late gene promoters at the time of the onset of late gene expression. The same results were obtained when myogenin and Mef2D were expressed in the absence of MyoD; however, these cells completed the morphological changes associated with skeletal muscle differentiation and formed myotubes, which the MyoD differentiated cells did not do. These contrasting results led to the realization that the muscle-specific isoform, Mef2D1b, was needed for promoting myotube formation, as myotubes formed in culture cells where it was

introduced, as well as in embryonic tissue, where it is highly induced during muscle development (Figure 6). However, myotubes did not form in cultured cells differentiated by MyoD because these cells did not induce the Mef2D1b isoform (Figure 6; Russo *et al*, 1998). The data strongly suggest that the Mef2D1b isoform plays a critical and specific role in the induction of gene products necessary to promote cell fusion and myotube formation.

Temporal control of skeletal muscle gene expression may be accomplished by differential recruitment of SWI/SNF chromatin-remodeling enzymes by different myogenic regulators

Although Brg1 and presumably the SWI/SNF enzymes are required for induction of myogenin and Mef2, the data indicate that a bypass of these induction events is not sufficient to promote myogenesis, as myogenin/Mef2D cannot direct changes in myogenic late gene chromatin accessibility or specify late gene expression in the absence of functional Brg1 (Figures 6 and 7). The physical interactions observed between endogenous Brg1 and Mef2 and myogenin proteins support the idea that Mef2 and myogenin are targeting the chromatin-remodeling enzymes to the late gene promoters. We therefore conclude that determination of skeletal muscle differentiation by myogenin and Mef2 occurs via recruitment of SWI/SNF chromatin-remodeling enzymes to late gene regulatory sequences.

Previous work has indicated that chromatin remodeling by SWI/SNF complexes is important for activation of the myogenin gene at early times of differentiation and that MyoD targets SWI/SNF to the myogenin promoter (de la Serna *et al*, 2001a, 2005; Simone *et al*, 2004). Multiple studies implicate the Pbx homeodomain protein as a cooperating regulator in the activation of myogenin by MyoD (Knoepfler *et al*, 1999; Berkes *et al*, 2004; de la Serna *et al*, 2005), and endogenous Pbx1 and MyoD can physically associate with Brg1 in differentiating cells (de la Serna *et al*, 2005). In contrast, MyoD binds to late gene promoters prior to the onset of late gene expression, but does not promote gene activation. Activation occurs upon induction of myogenin, which apparently replaces MyoD at late gene regulatory sequences, and concurrent induction of Mef2D, which acts as a cooperating regulator with myogenin. The observations that both the Pbx and Mef2 coregulators have the ability to physically interact with Brg1 suggests some parallels in the activation of early and late myogenic genes via recruitment of the SWI/SNF chromatin-remodeling enzymes.

The data indicate that selective recruitment of SWI/SNF enzymes by MRFs and their specific coregulators directly promotes alterations in chromatin structure at myogenic regulatory sequences and is a critical determinant of temporal control of myogenic gene expression.

Materials and methods

Preparation of mouse embryonic tissue

Pregnant C57 Bl/6 mice were killed at the indicated times of gestation and embryos were dissected as described (Hasty *et al*, 1993). Embryonic tissues were collected at embryonic days 10.5 (carcass; internal organs and head removed. At this stage, somites, but not myofibers, are present.), 12.5 (hind and fore limb buds), and 14.5 (hind and forelimb) as described (Venuti *et al*, 1995). For RNA isolation, tissue was homogenized and incubated in Trizol (Invitro-

gen). For ChIP and REAA, the dissected tissue was dissociated by fine mincing with microdissecting scissors, followed by treatment with collagenase (100 U/ml; Invitrogen) and dispase (17 U/ml; Invitrogen) for 30 min at 37°C while shaking vigorously. Enzymatic digestion was stopped by adding DMEM containing 20% fetal bovine serum. Digested tissues were washed twice with PBS, then used for REAA or fixed with 1% formaldehyde at room temperature for 10 min for ChIP.

Cell culture

The B22 cell line inducibly expressing dominant-negative BRG1 (de la Serna *et al*, 2000) was infected with retrovirus expressing MyoD or myogenin as described previously (Novitsch *et al*, 1996; Roy *et al*, 2002). Retrovirus expressing Mef2D1b was prepared by cloning full-length Mef2D1b (Martin *et al*, 1994) into the pBABE retroviral vector (Morgenstern and Land, 1990). Cells were cultured for 3 days in the presence of tetracycline (dominant-negative BRG1 repressed) or in the absence of tetracycline (dominant-negative BRG1 expressed), then passaged so that 24 h later the cells were 50% confluent. The cells were infected with the indicated retrovirus and incubated for 30 h. A low-serum differentiation media was then added to induce myogenic differentiation (de la Serna *et al*, 2001a). The time at which the differentiation media was added is referred to as time 0. Samples were collected at the times indicated (h) for analysis. Control samples were mock infected but still subjected to the differentiation protocol, and are labeled 'M' or 'mock' for mock differentiated.

Quantitative RT-PCR

For RT-PCR, total RNA (2 µg) was reverse transcribed with Superscript III RT (Invitrogen). qPCR was performed with Qiagen HotStart Taq Master Mix and Sybr GreenI as described previously (Schmittgen and Zakrajsek, 2000) using the following primers: MyoD, myogenin, In11, Myf5, skeletal α -actin, and Hprt primers (Valdez *et al*, 2000; Guidi *et al*, 2004). Other primers used were: Mef2A (5'-ATTT GAG AAG GAC TTT GTC CAT GTG A-3', 5'-CAATTT CAA CGG CAG CCC TGG ACT AG-3'), Mef2C (5'-GTT TCC TTC AAA CAC GTT TCC TTC T-3', 5'-CAG AGT TTG GAC AAC AAA GCC CTC AG-3'), Mef2D (5'-TCA TCAGTCCAACTTCCGCTTGTC-3', 5'-AAA TAT CAA CAA CCG TCG AGG CGG CT-3'), Mef2D1b (5'-AGA CGG AAG AGA AGT ATA AAA AGA TA-3', 5'-GCA GGC TCC ATT AGC ACT GTT GAG GT-3'), Brg1 (5'-CAT GGCCAATGCTGCT-3', 5'-CAGTACTCCCGCTCTTGT-3'), and EF1 α (5'-AGC TTC TCT GAC TAC CCT CCA CTT 3', 5'-GAC CGT TCT TCC ACC ACT GAT T-3'). Amplifications were performed in a DNA Engine Opticon System (MJ Research) and quantified. PCR reactions run on agarose gels were stopped in the linear range and visualized using SybrGreenI (Molecular Probes). mRNA levels were normalized to EF1- α mRNA levels at E10.5 (embryonic data) or to Hprt levels in each sample (cell line data). The data represent the mean plus/minus standard deviation from at least three independent experiments.

Antibodies, protein extracts, Western analysis, and immunoprecipitations

Isolation of protein and Western analyses were described (de la Serna *et al*, 2000). The coimmunoprecipitation experiments utilized a published protocol (Nerlov and Ziff, 1995). Antibodies used include myogenin (Santa Cruz, sc-576), Mef2 (Santa Cruz, sc-313; recognizes the Mef2A, C, and D isoforms), MyoD (Santa Cruz, sc-304), HDAC2 (Zymed, 51-5100), affinity-purified polyclonal rabbit antibody raised against GST fused to full-length MyoD (Lassar *et al*, 1989; de la Serna *et al*, 2005), and affinity-purified polyclonal rabbit antibody raised against GST fused to a unique portion of BRG1 (de la Serna *et al*, 2000).

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Chromatin immunoprecipitations

ChIPs were performed as described (de la Serna *et al*, 2005). Purified DNA from crosslinked cells was dissolved in 50 µl TE; 2 µl was used for PCR. Purified DNA from pooled embryos was dissolved in 50 µl TE; 2 µl was used for PCR. Inputs consisted of 1% of chromatin before immunoprecipitation. PCRs were performed with Qiagen HotStart Taq Master Mix using the following primer sets: IgH enhancer (de la Serna *et al*, 2005), MCK enhancer (5'-CCACTATGGGTCTAGGCTGCCCATGT-3' and 5'-TCTCTCCA TGCTGTACAGAGCCTAA-3'; –1253 to –1084), MCK promoter (5'-TGG TGA GCA TTA GCA ATG AAG GCA AT-3' and 5'-GTT CCT AAA CTA AGT GCT GAG TCT AG-3'; –401 to –207), desmin enhancer (5'-CTG TCA GCG TTC CTT GAC ACC TCT GTC-3' and 5'-ACC ACG GGT TTG TGT TTT GTC AGG AGA-3'; –979 to –779), and desmin proximal promoter (5'-GTC TTC TGT CCT CTT GGG GCT GTC CA-3' and 5'-TGG AGT GGA TGT GAA GAT GGG TGA C-3'; –199 to +10). Amplification was quantified by a DNA Engine Opticon System (MJ Research), then normalized to the input of each sample. PCR reactions run on agarose gels were stopped in the linear range and visualized using SybrGreenI. The Re-ChIP analysis was performed as described (Metivier *et al*, 2003).

Restriction enzyme accessibility assay

Restriction enzyme accessibility assays were performed as described (de la Serna *et al*, 2005). Briefly, cell nuclei were isolated with hypotonic buffer, washed, then incubated with 60 U of *PvuII*. The digested DNA was ligated with adaptor DNA using Takara ligation kit ver.2, following genomic DNA extraction from nuclei by DNeasy Tissue Kit (Qiagen). PCR amplification was performed to detect the appearance of the digested fragment arising from cleavage of MCK at –249 or Desmin at –98 using the sense primer LM-PCR1 (de la Serna *et al*, 2005), and antisense primers for MCK (5'-GTT CCT AAA CTA AGT GCT GAG TCT AG-3') or Desmin (5'-TGG AGT GGA TGT GAA GAT GGG TGA C-3'). Input was evaluated by using DNA purified from the *in vivo* restriction enzyme digest and PCR amplifying sequences proximal to the cleavage site. The sequences –1253 to –1200 (MCK) and –1032 to –862 (desmin) were amplified for this purpose. Amplification was quantified by a DNA Engine Opticon System (MJ Research), and then normalized to the input of each sample. PCR reactions run on agarose gels were stopped in the linear range and visualized using SybrGreenI.

Immunocytochemistry

B22 cells grown on coverslips were washed in PBS at 37°C for 2 min and fixed with 4% paraformaldehyde for 15 min at room temperature. After rapid washing in PBS, cells were permeabilized at 37°C for 10 min in 0.5% Triton X-100 in PBS, then incubated in blocking buffer as described (Molkentin *et al*, 1995). MyHC was detected with the MyHC hybridoma clone MF20 (Bader *et al*, 1982), followed by an Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) for 1 h at RT. Coverslips were mounted with Prolong (Molecular Probes). MyHC-expressing cells were visualized by confocal microscopy (Leica).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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